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(54) Title: MELANOCORTIN-4 RECEPTOR GENE AND USE AS A GENETIC MARKER FOR FAT CONTENT, WEIGHT GAIN, AND/OR FEED CONSUMPTION OF ANIMALS		
(57) Abstract Genetic markers in the porcine melanocortin-4 receptor (MC4R) gene are disclosed which are associated with fat content, growth rate, and feed consumption. Further, novel sequence data from regions of the gene are disclosed which may be used in a PCR test to screen for the presence of the marker. The genetic marker may be used to screen animals for breeding purposes which have the desired traits regarding fat content, growth rate, and feed consumption. Kits which take advantage of the PCR test are also disclosed.		

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TITLE: MELANOCORTIN-4 RECEPTOR GENE AND USE AS A GENETIC
MARKER FOR FAT CONTENT, WEIGHT GAIN, AND/OR FEED
CONSUMPTION OF ANIMALS

5 CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 60/094,287 filed July 27, 1998 and U.S. Provisional Application Serial No. 60/116,186, filed January 15, 1999, the disclosures of which are hereby incorporated by reference.

10 GRANT REFERENCE CLAUSE

This invention was supported at least in part by grants from the United States Department of Agriculture through the Iowa Agriculture and Home Economics Experiment Station (IaHees) and Project Number IOW03148 (Hatch Funds). The United States government may have certain rights in this invention.

15

FIELD OF THE INVENTION

The present invention relates to a method of genetically evaluating animals by assaying for the presence of at least one genetic marker which is indicative of one or more of the traits of fat content, growth rate, and feed consumption. In particular, the method
20 analyzes for variation in the melanocortin-4 receptor (MC4R) gene which is indicative of these traits. Even more particularly, the method analyzes for a polymorphism in the MC4R gene.

BACKGROUND OF THE INVENTION

25 There is an increasing consumer demand for meat products having low fat content. This demand is fueled by accumulating evidence in the scientific literature that a high consumption of animal fat, especially fat with a high proportion of saturated fatty acids, represents a significant health hazard, including risk for cardiovascular disease. Other health concerns associated with high fat meats include
30 their high content of cholesterol and the addition of relatively high amounts of salt which are added to improve the binding characteristics since salt aids in extracting the native

water binding component myosin from the meat. Furthermore, an increasing number of consumers find meat products containing chemical additives such as phosphates, emulsifying additives, and anti-oxidants less acceptable.

5 Faced with consumers who seek a healthier meat product, meat producers are continually pressed to offer cheaper and healthier products.

Cheaper products, of course, come from lowering costs of production. Producers are always interested in improving the growth rate and feed conversion of their animals. Lower production costs come from the shorter time to market and lower costs of feeding an animal. This can increase the profit margin in the livestock industry and/or result in lower
10 prices to the consumer.

By being able to select for animals which have the aforementioned traits, producers can raise animals with these desirable characteristics. Selection for desirable traits has traditionally been done using breeding techniques.

Genetic differences exist among individual meat producing animals as well as
15 among breeds which can be exploited by breeding techniques to achieve animals with these desirable characteristics. For example, Chinese breeds are known for reaching puberty at an early age and for their large litter size, while American breeds are known for their greater growth rates and leanness. Thus, it would be desirable to combine the best characteristics of both types of these breeds, thereby improving pork production.

20 Often, however, heritability for desired traits is low, for example, heritability for litter size is around 10%-15%. Standard breeding methods which select individuals based upon phenotypic variations do not take fully into account genetic variability or complex gene interactions which exist. Therefore, there is a need for an approach that deals with selection for leanness, growth rate, and feed consumption at the cellular or DNA level.

25 This method will provide a method for genetically evaluating animals to enable breeders to more accurately select those animals which not only phenotypically express desirable traits but those which express favorable underlying genetic criteria. This has largely been accomplished to date by marker assisted selection.

Restriction fragment length polymorphism (RFLP) analysis has been used by
30 several groups to study pig DNA. Jung et al., Theor. Appl. Genet., 77:271-274 (1989), incorporated herein by reference, discloses the use of RFLP techniques to show genetic

variability between two pig breeds. Polymorphism was demonstrated for swine leukocyte antigen (SLA) Class I genes in these breeds. Hoganson et al., Abstract for Annual Meeting of Midwestern Section of the American Society of Animal Science, March 26-28, 1990, incorporated herein by reference, reports on the polymorphism of swine major

5 histocompatibility complex (MHC) genes for Chinese pigs, also demonstrated by RFLP analysis. Jung et al., Animal Genetics, 26:79-91 (1989), incorporated herein by reference, reports on RFLP analysis of SLA Class I genes in certain boars. The authors state that the results suggest that there may be an association between swine SLA/MHC Class I genes and production and performance traits. They further state that the use of SLA Class I

10 restriction fragments, as genetic markers, may have potential in the future for improving pig growth performance.

The ability to follow a specific favorable genetic allele involves a novel and lengthy process of the identification of a DNA molecular marker for a major effect gene. The marker may be linked to a single gene with a major effect or linked to a number of genes

15 with additive effects. DNA markers have several advantages; segregation is easy to measure and is unambiguous, and DNA markers are co-dominant, i.e., heterozygous and homozygous animals can be distinctively identified. Once a marker system is established selection decisions could be made very easily, since DNA markers can be assayed any time after a tissue or blood sample can be collected from the individual infant animal.

20 The use of genetic differences in receptor genes has become a valuable marker system for selection. For example, United States Patents 5,550,024 and 5,374,526 issued to Rothschild et al. disclose a polymorphism in the pig estrogen receptor gene which is associated with larger litter size, the disclosure of which is incorporated herein by reference. United States application serial number 08/812,208 discloses polymorphic

25 markers in the pig prolactin receptor gene which are associated with larger litter size and overall reproductive efficiency.

It can be seen from the foregoing that a need exists for a method for selecting animals with the improved metabolic traits regarding fat content, growth rate, and feed consumption.

30

SUMMARY OF THE INVENTION

An object of the present invention is to provide a genetic marker based on or within the MC4R gene which is indicative of fat content, growth rate, and/or feed consumption.

Another object of the invention is to provide an assay for determining the presence
5 of this genetic marker.

A further object of the invention is to provide a method of evaluating animals that increases accuracy of selection and breeding methods for the desired traits.

Yet another object of the invention is to provide a PCR amplification test which will greatly expedite the determination of presence of the marker.

10 An additional object of the invention is to provide a kit for evaluating a sample of animal DNA for the identified genetic marker.

These and other objects, features, and advantages will become apparent after review of the following description and claims of the invention which follow.

This invention relates to the discovery of a polymorphism within the melanocortin-
15 4 receptor (MC4R) gene which is associated with fat content, growth rate, and feed conversion traits in animals. According to the invention, the association of the MC4R polymorphism with the trait(s) enables genetic markers to be identified for specific breeds or genetic lines. The *TaqI* restriction pattern which identifies the polymorphism is used to assay for the presence or absence of markers associated with the desirable metabolic traits.
20 The breed-dependent marker genotype (i.e., a marker in some breeds and a nonmarker in others) consists of a polymorphism within MC4R, a guanine to adenine transition at position 678 of the PCR product (a missense mutation of aspartic acid codon (GAU) into asparagine codon (AAU) at position 298 amino acid of the MC4R protein). The invention includes assays for detection of the marker as well as the sequence characterization of the
25 polymorphism and includes novel sequences in the MC4R gene which may be used to design amplification primers for such an assay. Additionally, the invention includes a method for using the assay in breeding programs for animal selection and a kit for performing the assay.

Definitions

30 As used herein, "low fat content" or "leanness" means a biologically significant decrease in body fat relative to the mean of a given population.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the sequence listing for MC4R in pigs (SEQ ID NO:1). "X" represents the site of the polymorphism.

5 Figure 2 represents a comparison of the DNA sequence between the human (SEQ ID NO:2) and the porcine (SEQ ID NO:3) MC4R gene.

Figure 3 represents a comparison of the amino acid sequence between the human (SEQ ID NO:4) and the porcine (SEQ ID NO:5) MC4R gene.

Figures 4a, 4b, and 4c are linkage reports for MC4R from CRI-MAP.

10 Figure 5 depicts partial nucleotide and amino acid sequences (SEQ ID NO:12) of the porcine MC4R gene. The amino acid translation shows an amino acid substitution at codon 298.

Figure 6 is an electrophoresis gel of *TaqI* digestion of the PCR product. Molecular marker (M) and MC4R genotypes are indicated at the top of each lane.

15 Figure 7 depicts multiple-alignments of the putative seventh transmembrane domain of porcine MC4R with other MCRs and GPCRs. The "*" represents the predicted sequence positions for porcine MC4R. The other amino acid sequences were obtained from the GenBank database (accession numbers P32245, P70596, P41983, P56451, P34974, P41968, P33033, Q01718, Q01726, Q28031, AF011466, P21554, P18089, P30680, P47211). The missense variant in porcine MC4R substituted amino acid N for D in the position marked with an arrow. The Asp (D) residue is highly conserved among MCRs, and the Asn (N) residue is well conserved in most other GPCRs.

20

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

25 Obesity is a disease affecting energy balance. The control of energy metabolism is simple: store excessive energy as fat and manage the energy to avoid superfluous energy storage, i.e., obesity. Although several genes and signaling systems have been implicated in obesity, there has been little known about the interconnection of energy homeostatic mechanism and genetic polymorphism. The melanocortin-4 receptor (MC4R) has been

30 shown to be an important mediator of long term weight homeostasis. MC4R antagonists can increase food intake and body weight during chronic administration. Skuladottir, G.V.,

et al., "Long term orexigenic effect of a novel melanocortin 4 receptor selective antagonist", British J. of Pharm., 126(1):27-34 (1999).

Lu et al. (1994) suggested that the melanocortin receptors are involved in controlling food intake and energy balance through studying its antagonism to the *agouti* obesity syndrome. Huszar et al. (1997) found that inactivation of the melanocortin-4 receptor gene (MC4R) resulted in a maturity onset obesity syndrome in mice and demonstrated a major role of MC4R protein in the regulation of energy balance related to the *agouti* obesity syndrome. In addition, the MC4R protein mediates the effects of leptin, one of the important signaling molecules in energy homeostasis (Seeley et al. 1997).

10 According to the present invention, a variant or polymorphism in the MC4R gene has been located, and this genetic variability is associated with phenotypic differences in the metabolic traits of fat content, growth rate, and/or feed consumption.

In one embodiment of the invention, an assay is provided for detection of presence of a desirable genotype. The assay involves amplifying the genomic DNA purified from blood, tissue, semen, or other convenient source of genetic material by the use of primers and standard techniques, such as the polymerase chain reaction (PCR), then digesting the DNA with a restriction enzyme (e.g., *Taq I*) so as to yield gene fragments of varying lengths, and separating at least some of the fragments from others (e.g., using electrophoresis).

20 The fragments may also be detected by hybridizing with a nucleotide probe (e.g., radio-labeled cDNA probes) that contains all or at least a portion of the MC4R gene cDNA sequence to the separated fragments and comparing the results of the hybridization with assay results for a gene sequence known to have the marker or a sequence known to not have the marker. Selection and use of probes for detection of MC4R sequences based on the known and disclosed MC4R sequences is generally known to those skilled in the art. The probe may be any sequence which will hybridize to the separated digestion products and allow for detection.

25 Another embodiment of the invention provides a kit for assaying the presence in a MC4R gene sequence of a genetic marker. The marker being indicative of inheritable traits of fat content, growth rate, and/or feed consumption. The kit in a preferred embodiment also includes novel PCR primers comprising 4-30 contiguous bases on either side of the

polymorphism to provide an amplification system allowing for detection of the *Taq I* polymorphism by PCR and *Taq I* digestion of PCR products. The preferred primers are SEQ ID NO:8 and SEQ ID NO:9.

5 A further embodiment comprises a breeding method whereby an assay of the above type is conducted on a plurality of gene sequences from different animals or animal embryos to be selected from and based on the results, certain animals are either selected or dropped out of the breeding program.

According to the invention, the polymorphism in the MC4R gene, identifiable by the *Taq I* restriction pattern, is disclosed. As is known in the art, restriction patterns are not
10 exact determinants of the size of fragments and are only approximate. The polymorphism is identifiable by three bands from a *Taq I* digestion of the PCR product, 466, 225, and 76 base pairs (bp) for one homozygous genotype (allele 1); two bands, 542 and 225 bp for another homozygous genotype (allele 2); and four bands for the heterozygous genotype (542, 466, 225, and 76 bp). The marker for leanness and lower feed intake is identifiable
15 by the 466/225/76 bands, except for the Chinese pigs, where the Chinese pigs' marker for leanness is the 542/225 bands. The marker for faster rate of gain is identifiable by the 542/225 bands.

In addition, the polymorphism associated with the pattern has been identified at the nucleotide level. The polymorphic *Taq I* site was sequenced along with the general
20 surrounding area. See SEQ ID NO: 1. The sequences surrounding the polymorphism have facilitated the development of a PCR test in which a primer of about 4-30 contiguous bases taken from the sequence immediately adjacent to the polymorphism is used in connection with a polymerase chain reaction to greatly amplify the region before treatment with the *Taq I* restriction enzyme. The primers need not be the exact complement; substantially
25 equivalent sequences are acceptable.

From sequence data, it was observed that in allele 2 the guanine is substituted with an adenine at position 678 of the PCR product or at position 298 amino acid of the MC4R protein changing the aspartic acid codon (GAU) into an asparagine codon (AAU). The PCR test for the polymorphism used a forward primer of 5'-TGG CAA TAG CCA AGA
30 ACA AG-3' (SEQ. ID NO: 6) and a reverse primer of 5'-CAG GGG ATA GCA ACA GAT GA-3' (SEQ. ID NO: 7). Pig specific primers used were a forward primer of 5'-TTA

AGT GGA GGA AGA AGG-3' (SEQ. ID NO: 8) and a reverse primer of 5'-CAT TAT GAC AGT TAA GCG G-3' (SEQ ID NO:9). The resulting amplified product of about 750 bp, when digested with *Taq I*, results in allelic fragments of 466, 225, and 76 bp (allele 1) or 542 and 225 bp (allele 2).

5 The marker may be identified by any method known to one of ordinary skill in the art which identifies the presence or absence of the marker, including for example, single-strand conformation polymorphism analysis (SSCP), RFLP analysis, heteroduplex analysis, denaturing gradient gel electrophoresis, and temperature gradient electrophoresis, ligase chain reaction or even direct sequencing of the MC4R gene and examination for the
10 *Taq I* RFLP recognition pattern.

One or more additional restriction enzymes and/or probes and/or primers can be used. Additional enzymes, constructed probes, and primers can be determined by routine experimentation by those of ordinary skill in the art.

Other possible techniques include non-gel systems such as TaqMan™ (Perkin
15 Elmer). In this system, oligonucleotide PCR primers are designed that flank the mutation in question and allow PCR amplification of the region. A third oligonucleotide probe is then designed to hybridize to the region containing the base subject to change between different alleles of the gene. This probe is labeled with fluorescent dyes at both the 5' and 3' ends. These dyes are chosen such that while in this proximity to each other the
20 fluorescence of one of them is quenched by the other and cannot be detected. Extension by *Taq* DNA polymerase from the PCR primer positioned 5' on the template relative to the probe leads to the cleavage of the dye attached to the 5' end of the annealed probe through the 5' nuclease activity of the *Taq* DNA polymerase. This removes the quenching effect allowing detection of the fluorescence from the dye at the 3' end of the probe. The
25 discrimination between different DNA sequences arises through the fact that if the hybridization of the probe to the template molecule is not complete, i.e., there is a mismatch of some form, the cleavage of the dye does not take place. Thus, only if the nucleotide sequence of the oligonucleotide probe is completely complementary to the template molecule to which it is bound will quenching be removed. A reaction mix can
30 contain two different probe sequences each designed against different alleles that might be present, thus, allowing the detection of both alleles in on reaction.

Though the use of RFLPs is one method of detecting the polymorphism, other methods known to one of ordinary skill in the art may be used. Such methods include ones that analyze the polymorphic gene product and detect polymorphisms by detecting the resulting differences in the gene product.

5 Though the preferred method of separating restriction fragments is gel electrophoresis, other alternative methods known to one skilled in the art may be used to separate and determine the size of the restriction fragments.

 It is possible to indirectly select for the polymorphism with alternative DNA markers. It is possible to establish a linkage between specific alleles of alternative DNA markers and alleles of DNA markers known to be associated with the MC4R gene which
10 have previously been shown to be associated with a particular trait. Examples of markers on the published PiGMap chromosome map which are linked to the MC4R gene include S0331, BHT0433, and S0313.

 The reagents suitable for applying the methods of the present invention may be
15 packaged into convenient kits. The kits provide the necessary materials, packaged into suitable containers. At a minimum, the kit contains a reagent that identifies the polymorphism in the MC4R gene that is associated with the traits of interest, fat content, growth rate, and feed consumption. Preferably, the reagent that identifies the polymorphism is a PCR set (a set of primers, DNA polymerase, and four nucleoside
20 triphosphates) that hybridize with the MC4R gene or a fragment thereof. Preferably, the PCR set and restriction enzyme that cleaves the MC4R gene in at least one place are included in the kit. Preferably, the kit further comprises additional means, such as reagents, for detecting or measuring the detectable entity or providing a control. Other reagents used for hybridization, prehybridization, DNA extraction, visualization, and
25 similar purposes may also be included, if desired.

 The genetic markers, methods, and kits of the invention are useful in a breeding program to improve fat content, growth rate, and feed consumption characteristics in a breed, line, or population of animals. Continuous selection and breeding of animals that are at least heterozygous and preferably homozygous for the desired polymorphism
30 associated with the particular trait would lead to a breed, line, or population having those desired traits. Thus, the marker is a selection tool.

The following examples are offered to illustrate, but not limit the invention.

EXAMPLE 1

5 Melanocortin 4 Receptor PCR-RFLP Test - *TaqI* polymorphism and Genetic Linkage Mapping of MC4R Gene

Primers:

Primers were designed from homologous regions of human and rat MC4R
 10 sequences (Genbank Accession No. s77415 and u67863, respectively). These primers were used to amplify a 750-bp sequence of the porcine MC4R gene.

MC4R1: 5' TGG CAA TAG CCA AGA ACA AG 3' (SEQ ID NO:6)

MC4R4: 5' CAG GGG ATA GCA ACA GAT GA 3' (SEQ ID NO:7)

15

PCR Conditions:

Mix 1:	10X Promega Buffer	1.0 μ L
	25 mM MgCl ₂	0.6 μ L
	dNTPs mix (2.5mM each)	0.5 μ L
	25 pmol/ μ L MC4R1	0.1 μ L
	25 pmol/ μ L MC4R4	0.1 μ L
	dd sterile H ₂ O	7.5 μ L
	<i>Taq</i> Polymerase (5 U/ μ L)	0.07 μ L
	Genomic DNA (12.5 ng/ μ L)	1.0 μ L

Ten μ L of Mix 1 and DNA were combined in reaction tube, then overlaid with mineral oil. The following PCR program was run: 94°C for 2 min.; 35 cycles of 94°C for
 20 30 sec.; 58°C 1 min., and 72°C 1 min. 30 sec.; followed by a final extension at 72°C for 15 min.

Five μL of the PCR reaction product was checked on a standard 1% agarose gel to confirm amplification success and clean negative control. Product size is approximately 750 base pairs. Digestion was performed by the following procedure.

<u>TaqI Digestion Reaction</u>	<u>10 μL reaction</u>
PCR product	5.0 μL
10X TaqI NE Buffer	1.0 μL
BSA (10mg/ml)	0.1 μL
TaqI enzyme (20 U/ μL)	0.5 μL
dd sterile H ₂ O	3.4 μL

5

A cocktail of the buffer, enzyme, BSA, and water was made. Five μL was added to each reaction tube containing the DNA. The mixture was then incubated at 65°C for at least 4 hours to overnight. Loading dye was mixed with the digestion reaction and the total volume was loaded on a 3% agarose gel. The major bands for allele 1 are about 466, 225, and 76 bp. The allele 2 genotype bands are 542 and 225 bp. The heterozygote genotype has both allele 1 and allele 2.

10

Results

The amplified PCR product is about 750 bp. The sequence of the PCR product confirmed that the PCR product is MC4R gene with 97.6%, and 92.2% identities at the amino acid and DNA level, respectively, to corresponding human sequences. (see Figs. 2 and 3).

15

The TaqI digestion of the PCR product produced allelic fragments of 466, 225, and 76 bp (allele 1), or 542 and 225 bp (allele 2). The heterozygote genotype has both types of alleles. Mendelian inheritance was observed in three three-generation international reference families, which were used to map this gene by linkage analysis.

20

The polymorphism between allele 1 and allele 2 resulting from a G \rightarrow A transition at position 678 of the PCR product revealed a missense mutation of Aspartic acid codon (GAU) into Asparagine codon (AAU) at position 298 amino acid of MC4R protein. (See Figure 1, SEQ ID NO:1).

25

Allele frequencies were determined by genotyping of DNA samples from a small number of animals from different breeds (Table 1). Allele 1 was observed with a frequency of 1 in Meishan, but was not observed or observed at very low frequency in Hampshire and Yorkshire. The frequencies of allele 1 in Landrace and Chester White were 0.5, respectively.

Figures 2 and 3 illustrate the differences between the DNA and amino acid sequences of the human and porcine MC4R gene (SEQ ID NOS:2-5).

TABLE 1
The Frequency of Allele 1 in Different Pig Breeds

Breed	# Animals	Freq. Allele 1
Meishan	8	1
Large White	8	0.56
Yorkshire	6	0.08
Hampshire	5	0
Landrace	4	0.5
Chester White	4	0.5
Minzu	2	1
Wild Boar	2	1

Linkage Analyses

Two-point and multi-point linkage analyses were performed on the genotypes of international reference families. See Figs. 4a-4c. The data were analyzed by using the CRI-MAP program. MC4R was significantly linked to several markers on porcine chromosome (SSC) 1. The most closely linked markers (recombination fraction and LOD score in parentheses) are SO331 (0.02, 21.97), BHT0433 (0.02, 21.32), and SO313 (0.00, 17.76) by two-point linkage analysis. A multi-point linkage analysis produced the best map order of markers and MC4R (with distance in Kosambi cM): KGF-5.8-CAPN3-2.5-MEF2A-6.1-MC4R-5.6-SO313.

Somatic cell hybrid panel of pig and rodent was used to assign MC4R to a cytogenetic region. PCR products from pig specific primers were amplified in clones 7, 8, 16, 18, and 19. MC4R was localized to SSC1q 22-27.

5

EXAMPLE 2

MC4R Receptor PCR-RFLP Test using Pig Specific Primers and Genetic Linkage Mapping of the Porcine MC4R Gene

Pig Specific Primer Sequences

10

Forward primer: 5'-TTA AGT GGA GGA AGA AGG-3' (SEQ ID NO:8)

Reverse primer: 5'-CAT TAT GAC AGT TAA GCG G-3' (SEQ ID NO:9)

Method of Detection

The PCR reaction was performed using

15

Porcine genomic DNA	12.5 ng
1x PCR buffer	
MgCl ₂	1.5 mM
dNTP	0.125 mM
Forward primer	0.3 μM
Reverse primer	0.3 μM
Taq DNA polymerase (Promega)	0.35 U

in a 10 μL final volume. The PCR profile included 2 min. at 94°C; 35 cycles of 30 sec. at 94°C, 1 min. at 56°C, 1 min. 30 sec. at 72°C; and 15 min. at 72°C in a Robocycler (Statagene, La Jolla, CA). A 5.0 μL aliquot of the PCR products was digested in a total
20 volume of 10 μL with 10 U of *TaqI* incubated overnight at 65°C. The digestion products were electrophoresed on a 3% agarose gel.

Description of Polymorphism

The *TaqI* digestion of the PCR product produced fragments of 466, 225, and 76 bp in allele 1 and 542 and 225 bp in allele 2. The heterozygous genotype has fragments of both allele 1 and allele 2.

5

Pattern of Inheritance

Autosomal segregation of Mendelian inheritance was observed in three three-generation European PiGMap families (Archibald et al., 1995).

10 **Allele Frequencies**

Allele frequencies were determined by genotyping the grandparental animals of the European PiGMap families and unrelated animals from ISU reference families. Allele 1 was observed with the following frequencies.

15

TABLE 2

The Frequency of Allele 1 in Different Pig Breeds

Breed	# Animals	Freq. Allele 1
Meishan	8	1
Large White	8	0.56
Yorkshire	10	0.15
Hampshire	12	0
Landrace	8	0.56
Chester White	8	0.56
Minzu	2	1
Wild Boar	2	1

Chromosomal Location

20 Two-point and multi-point linkage analysis were performed on the genotypes of three PiGMap families using the CRI-MAP program (Green et al. 1990). MC4R was

significantly linked to several markers on porcine chromosome 1 (SSC 1). The most closely linked markers (recombination fraction and LOD score in parentheses) are SO331 (0.02, 21.97), BHT0433 (0.02, 21.32), and SO313 (0.00, 17.76) according to two-point linkage analysis. The best map order of MC4R with respect to other linked markers produced by multi-point linkage analysis is as follows (with distance in Kosambi cM): KGF-5.8-CAPN3-2.5-MEF2A-6.1-MC4R-5.6-SO313.

Comments

The Melanocortin-4 Receptor is a G protein-coupled, seven-transmembrane receptor expressed in the brain. Huszar et al. (1997) found that inactivation of MC4R gene resulted in a maturity onset obesity syndrome in mice and demonstrated a major role of MC4R protein in the regulation of energy balance. The MC4R gene has been mapped to human chromosome 18q21.3 (Gantz et al., 1993). The localization of MC4R gene to SSC 1 is consistent with previous chromosome painting data indicating synteny between this chromosome and HSA 18 and 15 (Goureau et al., 1996). However, the gene order of several genes previously mapped from HSA 18 and 15 to SSC 1, including CAPN3, KGF, and MEF2A, is not conserved with MC4R. Therefore, mapping of MC4R to SSC 1 may identify an evolutionary breakpoint between HSA 18 and 15 in relation to SSC 1.

EXAMPLE 3

Association of Marker with Enhanced Metabolic Characteristics

In a preliminary study to determine which allele is associated with which trait and in which breeds, the genotypes of several lines of animals were correlated with days to 110 kg, backfat measurements, daily gains, and average daily feed intake. The pigs used in the study were from lines from Pig Improvement Company (PIC).

Data was accumulated using the PCR test described *supra* for the 1 and 2 allele of the MC4R gene. The collected data is summarized in Tables 3-8 below.

According to the results, allele 1 is the significantly leaner allele (see P2 backfat measurements) in all lines except in Chinese pigs where it is the fat allele. Allele 2 is

associated with significantly faster rate of gain (test daily gain) in the tested commercial lines. Overall allele 1 is associated with lower feed intake.

TABLE 3

5 Number of observations

MC4R genotype	L02	L03	L19	L65	Overall	L95
11	88	30		32	150	20
12	57	54	56	74	241	67
22	12	31	254	33	330	37
Total					721	

MC4R genotype:

11 = homozygous allele 1

12 = heterozygous

10 22 = homozygous allele 2

TABLE 4

Number of observations (males/females)

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	9/79	12/18		15/17	36/114		0/20
12	9/48	37/17	12/44	44/30	102/139		0/67
22	3/9	28/3	89/165	21/12	141/189		0/37

15

TABLE 5

Days to 110kg

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	169.7	172.4		169.6	168.5		219.1
12	170.2	171.5	165.0	171.2	168.7		212.2
22	165.3	173.4	162.9	170.3	167.1		211.4
	P<.23	P<.75	P<.15	P<.76	P<.31		P<.27

5

TABLE 6

P2 backfat (mm)

MC4R Genotype	L02	L03	L19	L65	Overall		L95
11	10.8	11.9		9.7	11.1		22.8
12	11.3	12.5	12.2	10.5	11.8		21.5
22	12.1	12.7	12.6	10.7	12.1		20.3
	P<.10	P<.43	P<.34	P<.17	P<.006		P<.17

TABLE 7

10 Test daily gain (gm/d)

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	882.2	811.0		881.8	871.9		688.8
12	891.2	820.5	875.6	873.0	876.3		676.2
22	969.1	819.5	906.7	906.2	906.9		692.5
	P<.01	P<.96	P<.05	P<.24	P<.006		P<.66

TABLE 8

Average daily feed intake (kg/d), boars only, except L95 which was gilts only

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	2.31	1.78		1.75	1.89		2.05
12	2.11	1.90	1.97	1.90	1.96		2.03
22	2.15	1.97	2.00	1.97	2.02		2.08
	P<.84	P<.14	P<.56	P<.14	P<.16		P<.36

5

EXAMPLE 4

A Missense Variant of the Porcine Melanocortin-4 Receptor (*MC4R*) Gene is Associated with Fatness, Growth, and Feed Intake Traits

To determine if there was an association of this *MC4R* polymorphism with phenotypic variation the mutation was tested in a large number of individual animals from several different pig lines. Analyses of growth and performance test records showed significant associations of *MC4R* genotypes with backfat, growth rate and feed intake in a number of lines. It is probable that the variant amino acid residue of the *MC4R* mutation causes a significant change of the *MC4R* function. These results support the functional significance of a pig *MC4R* missense mutation and suggest that comparative genomics based on model species may be equally important for application to farm animals as they are for human medicine.

Identification of mutations in the *leptin* and the *leptin receptor* has provided some information on genetic components involved in the regulation of energy balance (Zhang et al. 1994; Tartaglia et al. 1995). Genetic studies using animal models have facilitated the identification of major genetic causes of obesity (Andersson 1996; Pomp 1997; Giridharan 1998). Furthermore, several other genes involved in the neural signaling pathway of energy homeostasis have been identified (Flier and Maratos-Flier 1998; Schwartz et al. 1999). Of particular interest among candidate signaling molecules involved in the

regulation of energy homeostasis is the melanocortin-4 receptor (MC4R). The MC4R response to leptin signaling is a link between food intake and body weight (Seeley et al. 1997; Marsh et al. 1999). Neuropeptide Y (NPY) signaling in the central nervous system is also mediated by the MC4R protein (Kask et al. 1998). Several mutations in MC4R including frameshift and nonsense mutations are associated with dominantly inherited obesity in humans (Vaisse et al. 1998; Yeo et al. 1998). Some other MC4R missense mutations in humans have also been identified (Gotoda et al. 1997; Hinney et al. 1999) but the functional significance of these mutations has not been characterized.

Selection based on growth characteristics has been of great importance to the pig industry because of costs associated with feeding and consumer preference for lean meat. Efficient genetic improvement in these quantitative traits may be augmented through the use of marker assisted selection (MAS) using high density genetic maps (Dekkers and van Arendonk 1998; Rothschild and Plastow 1999). An important tool in this process is comparative mapping using the well-developed human and mouse gene maps, which assist in the identification of corresponding genomic regions or major genes controlling growth and performance traits in the pig. Biological understanding of complex traits in human or model species offers an alternative approach to identify genes responsible for the traits of economic interest in livestock. Several quantitative trait loci (QTL) linkage scans using phenotypically divergent breeds and candidate gene analyses have been successfully conducted for fatness and growth traits (Yu et al. 1995; Casas-Carrillo et al. 1997; Knorr et al. 1997; Knott et al. 1998; Rohrer et al. 1998; Wang et al. 1998; Paszek et al. 1999), but no individual genes with major effects on growth and performance traits have yet been established for commercial populations. The role of MC4R in feed intake and obesity suggests it may be an important genetic marker for the growth-related traits in the pig.

Materials and Methods

Animals. Pigs were raised under normal production conditions under the care of PIC employees in nucleus farms in the United States and Europe. Pigs were put on the performance test at approximately 70 days of age and taken off test after 13 weeks. At the end of the trial backfat was measured ultrasonically in real time (B mode) at the 10th rib 2 cm from the centerline. Average daily gain (growth) over the test period was calculated as

weight gained divided by days on test. Days to 110 kg market weight was estimated using standard procedures and feed intake was measured using individual electronic measurement equipment.

5 PCR amplification of a pig MC4R gene fragment. Primers were designed from homologous regions of human and rat MC4R sequences (GenBank accession no. s77415 and u67863, respectively). The primers were: forward primer: 5'-TGG CAA TAG CCA AGA ACA AG-3' (SEQ. ID NO:6) and reverse primer: 5'-CAG GGG ATA GCA ACA GAT GA-3' (SEQ. ID NO:7). The PCR reaction was performed using 12.5 ng of porcine
10 genomic DNA, 1x PCR buffer, 1.5 mM MgCl₂, 0.125 mM dNTPs, 0.3 mM of each primer, and 0.35 U *Taq* DNA polymerase (Promega) in a 10 μ L final volume. The conditions for PCR were as follows: 2 min at 94°C; 35 cycles of 30 s at 94°C, 1 min at 56°C, 1 min 30 s at 92°C, and a final 15 min extension at 72°C in a Robocycler (Stratagene, La Jolla, CA).

15 Sequencing and mutation detection. Sequencing of the PCR products from several individual pigs of different breeds was conducted and the sequences were compared to detect any nucleotide change. Sequencing was performed on an ABI sequencer 377 (Applied Biosystems). The porcine MC4R sequence has been submitted to GenBank, and has accession number AF087937. The sequence analysis revealed one nucleotide
20 substitution situated within a *TaqI* restriction enzyme recognition site (Kim et al. 1999). A set of primers was then designed to generate a smaller MC4R gene fragment, which contained only one informative *TaqI* restriction site to specify the polymorphic site and to facilitate the PCR-RFLP test. These primers were: forward 5'-TAC CCT GAC CAT CTT GAT TG-3' (SEQ. ID NO:10) and reverse: 5'-ATA GCA ACA GAT GAT CTC TTT G-3'
25 (SEQ. ID NO:11).

Statistical analysis. Analysis of variance procedures were used with a mixed model that accounted for the fixed effects of farm, test period, sex of the animal, the MC4R genotype and site (random). All animals in lines of American/European descent (Lines A-
30 D) were pooled for the overall analysis and in this analysis line of origin was included.

Mean effects were estimated for each genotype and are presented in Tables 9-15. Overall F tests were used to determine level of significance.

Results

5 Identification of a missense mutation in the pig MC4R gene. The MC4R gene consists of approximately 1 kb of coding sequence contained within a single exon. About 750 bp of a pig MC4R gene fragment was produced by PCR (Kim et al. 1999). The sequence of the PCR product confirmed that the PCR product is the MC4R gene with 92.2% and 97.6% identities at nucleotide and the amino acid levels, respectively, to the
10 human MC4R sequence. Multiple alignments of the sequences from individual animals of several breeds identified a single nucleotide substitution (G→A; Fig. 5). The polymorphism revealed a missense mutation that replaces aspartic acid (GAU) with asparagine (AAU) at the position identical to amino acid 298 of human MC4R protein. To confirm this base change, we designed pig-specific primers flanking the polymorphic site
15 and analyzed the polymorphism as a *TaqI* PCR-RFLP gel (Fig. 6). Figure 6 shows a *TaqI* digestion of the PCR product analyzed by agarose-gel electrophoresis. Allele 1 produced 156 and 70 bp fragments and allele 2 produced a 226 bp fragment as the PCR-RFLP. The heterozygote has both allele 1 and 2 fragments. Molecular marker (M) and MC4R genotypes are indicated at the top of each lane.

20

The MC4R missense mutation is within a highly conserved region among melanocortin receptors (MCR). The MCR is a subfamily of G-protein coupled receptors (GPCR) containing certain conserved structural elements common to most other GPCRs, but overall amino acid identities between MCR and other GPCRs are low (Tatro 1996). A
25 multiple-alignment of the predicted amino acid sequences of the pig MC4R with MC4R proteins from other species, other MCR proteins, or representative GPCRs showed that the aspartic acid found at position 298 of the seventh transmembrane domain is very highly conserved in the MCR proteins (Fig. 7). It is interesting to note, however, that this position is occupied by asparagine in most other GPCRs. The MCR proteins show 40-80% amino
30 acid identity with each other (Tatro 1996), but the second intracytoplasmic loop and the seventh transmembrane domain are highly conserved among MCR proteins (Gantz et al.

1993). Some of the relationships between MCR structure and function have been discovered by the studies of natural and experimental mutations in humans and mice (Robbins et al. 1993; Valverde et al. 1995; Frandberg et al. 1998). These studies indicate that some mutations in highly conserved regions cause structural changes and alter the function of the receptor. The Asp298Asn substitution mutation could have an effect on the function of the receptor. However, this will require further testing but it is known that change of the homologous residue in MC1R (Asp294His) is associated with fair skin and red hair in humans (Valverde et al. 1995).

10 The MC4R missense mutation is associated with obesity-related traits. To investigate the effects of the missense mutation, the relationship of MC4R genotypes was analyzed for the effects on variation in growth rate, backfat, and feed consumed in over 1,800 animals from several commercial pig lines from PIC, an international pig breeding company. The animals were from closed commercial lines of European/American breeds (Lines A-D) together with a line originating from a cross between a European and a Chinese breed (Line E). In lines A-D significant associations of the MC4R genotypes were found for all performance traits. The animals homozygous for allele 1 had on average significantly less backfat ($P < .001$), lower daily gain ($P < .001$), and lower feed intake ($P < .01$) than those of the homozygous 22 genotype animals (Tables 11, 13, & 15). Overall, pigs with the 11 genotype had approximately 9% less backfat than pigs with the 22 genotype (Table 11), whereas pigs with the 22 genotype grow significantly faster (37g/day) than pigs with the 11 genotype (Table 13). These results appear to be a function of appetite because the 22 genotype animals consume considerably more feed (Table 15). The association between the missense variant of the MC4R gene and related performance traits is clearly established in European/American breeds. Although the number of tested animals is much smaller, these results were not seen in the considerably fatter Chinese crossed line (Line E). Interestingly, line E shows a trend for backfat in the opposite direction to that observed in the other lines (Table 11).

Discussion

The present study clearly demonstrates that the porcine MC4R missense mutation is significantly associated with several performance traits in pigs. Allele 1 representing Asp298, the well conserved amino acid within other MCR subtypes and other species MC4R, was associated with less backfat thickness, slower growth rate, and lower feed intake and allele 2 representing Asn298 was associated with fatter, higher feed intake, and faster growing animals. As the highly conserved residues in the melanocortin receptor proteins have important roles for ligand binding or intracellular signal transmission (Tatro 1996), the MC4R variants might exert functionally distinct abilities in the regulation of food intake and body weight. Further testing of this hypothesis will provide important insights into the structural basis of MCR function and a molecular target for the treatment of human obesity.

Allele 1 was associated with the fattest animals in Line E, which was derived by crossing a Chinese Large White breed with a line of Meishan origin. This is surprising given that the mutation causes a significant amino acid change in a well-conserved region. The result may be due to sampling. However, if we assume that this result will be significant when more results are added there are several possible explanations. One possibility could be the difference in the background gene effects (epistasis). As growth and fatness are complex polygenic traits, it is certainly possible that the Chinese breed has some distinct allelic interactions derived from several hundred years of isolation and these putative interaction(s) might create variation in polygenic traits within crosses between widely different lines (Frankel and Schork 1996). Several QTL analyses have been conducted for fatness and growth traits using divergent lines (Cases-Carrillo et al. 1997; Knott et al. 1998; Rohrer et al. 1998; Wang et al. 1998; Paszek et al. 1999), but QTL have not been reported near the MC4R locus, which maps to chromosome 1 at approximately 80 cm on the linkage map (data not shown). It may mean that the epistatic effects of the MC4R alleles suggested in Line E have made it difficult to observe the MC4R locus in most QTL experiments which have involved crosses between Chinese and European/American lines. It is likely that the effect of some alleles will be variable in the different backgrounds and hard to detect in QTL experiments involving genetically divergent breeds.

The effect of MC4R variant will possibly be explained by further studies on the biological effect caused by this mutation in other pig breeds and lines. However, given the strong relationship of MC4R variants to leanness, growth and feed intake, this mutation could be used immediately for marker assisted selection (Meuwissen and Goddard 1996) to develop lines of pigs to satisfy particular customer requirements. For instance, in sow lines where appetite is normally decreased after farrowing, selection for the MC4R 2 allele could help improve feed intake. Furthermore, in some lines deemed to be too fat, selection for allele 1 could be employed and in lines that were considered too slow growth allele 2 selection could be also employed. Therefore, genotyping for the MC4R mutation in pig breeding lines will improve the selection efficiency of feed related production traits including growth and leanness. The candidate gene approach has also been used for investigating the role of the porcine leptin gene (Jiang and Gibson 1999). However, in the leptin case, although there was evidence for an association between a leptin polymorphism and backfat depth in a cross between a commercial breed and an unimproved line, there was no clear association in the different commercial lines tested (Jiang and Gibson 1999). Therefore, it should not be assumed that since one finds a gene that one can assume a relationship exists. In contrast, with MC4R we have determined that variation in this candidate gene can explain significant variation for backfat, growth rate, and feed intake in commercial lines of pigs. These results with MC4R illustrate the potential value of comparative genetic analyses using candidate genes in livestock genomics.

EFFECT OF MC4R GENOTYPE ON SEVERAL PRODUCTION TRAITS IN THE PIG

TABLE 9

Number of observations (males/females/totals) for Days to 110 kg and backfat

MC4 R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	9/212/221	12/94/106		37/17/54	58/323/381	0/20/20
12	9/150/159	37/96/133	12/158/170	152/30/182	210/434/644	0/67/67
22	3/16/19	28/36/64	89/356/445	155/12/167	275/420/695	0/37/37

TABLE 10

Days to 110kg

MC4 R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	166.3+/-0.8	168.4+/-1.4		170.0+/-2.4	167.9+/-0.9	219.1+/-4.8
12	165.6+/-0.9	166.8+/-1.1	163.9+/-1.0	170.2+/-1.8	166.9+/-0.8	212.2+/-3.4
22	162.3+/-2.3	166.8+/-1.5	161.5+/-0.8	167.0+/-1.9	164.6+/-0.9	211.4+/-4.0
	P <.24	P <.47	P <.007	P <.10	P <.001	P <.27

5

TABLE 11

10th rib Backfat (mm)

MC4 R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	10.7+/-0.2	12.1+/-0.2		9.8+/-0.5	11.1+/-0.2	22.8+/-1.2
12	11.2+/-0.2	12.5+/-0.2	12.3+/-0.2	10.5+/-0.4	11.6+/-0.2	21.5+/-0.9
22	12.5+/-0.5	12.6+/-0.3	12.7+/-0.2	10.9+/-0.4	12.0+/-0.2	20.3+/-1.0
	P <.02	P <.31	P <.06	P <.05	P <.001	P <.17

TABLE 12

Number of observations (males/females/totals for Test daily gain

MC4 R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	9/105/114	12/38/50		37/17/54	58/160/218	0/20/20
12	9/65/74	37/35/72	12/97/109	152/30/182	210/227/437	0/67/67
22	3/13/15	28/15/43	89/225/314	155/12/167	275/265/539	0/37/37

10

TABLE 13

Test daily gain (gm/day)

MC4 R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	892.6+/-10.4	841.7+/-13.8		882.2+/-18.4	871.9+/-10.2	688.8+/-24.5
12	913.3+/-11.6	868.4+/-12.1	882.2+/-12.9	883.7+/-14.3	885.1+/-8.9	676.2+/-17.6
22	982.8+/-22.8	862.4+/-15.1	913.4+/-10.5	904.6+/-15.1	908.8+/-9.3	692.5+/-20.4
	P <.001	P <.28	P <.006	P <.20	P <.001	P <.66

TABLE 14

5 Number of observations (males/females/total) for average daily feed intake

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	7/0/7	11/0/11		13/0/13	31/0/31	0/18/18
12	8/0/8	31/0/31	9/0/9	34/0/34	82/0/82	0/63/63
22	3/0/3	25/0/25	74/0/74	16/0/16	118/0/118	0/32/32

TABLE 15

Average daily feed intake (kg/day), boars only except LINE E which was gilts only

MC4 R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	2.31+/-0.2	1.78+/-0.09		1.75+/-0.06	1.94+/-0.07	2.05+/-0.10
12	2.11+/-0.3	1.90+/-0.07	1.97+/-0.10	1.90+/-0.07	2.03+/-0.06	2.03+/-0.07
22	2.15+/-0.4	1.97+/-0.06	2.00+/-0.07	1.97+/-0.08	2.11+/-0.06	2.08+/-0.08
	P <.84	P <.14	P <.56	P <.14	P <.01	P <.36

10

Having described the invention with reference to particular compositions, theories of effectiveness, and the like, it will be apparent to those of skill in the art that it is not intended that the invention be limited by such illustrative embodiments or mechanisms, and that modifications can be made without departing from the scope or spirit of the

invention, as defined by the appended claims. It is intended that all such obvious modifications and variations be included within the scope of the present invention as defined in the appended claims. The claims are meant to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless
5 the context specifically indicates to the contrary.

The following citations are hereby incorporated in their entirety by reference:

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- 10 fatness in four pig breeds" Mamm. Genome 12:191-193.

What is claimed is:

1. A method of identifying an animal which possesses a genotype indicative of the metabolic traits of fat content, growth rate, and feed consumption, the method comprising:
 - 5 a) obtaining a nucleic acid sample from the animal, and
 - b) identifying a polymorphism in the MC4R gene of the sample.
2. The method of claim 1 wherein the polymorphism is characterized by a nucleotide position 678 of the PCR product of the MC4R gene.
- 10 3. The method of claim 1 wherein the animal is a pig.
4. The method of claim 2 wherein the polymorphism at the nucleotide position 678 is associated with fat content.
- 15 5. The method of claim 2 wherein a guanine at the nucleotide position 678 is associated with lower feed intake.
6. The method of claim 2 wherein an adenine at the nucleotide position 678 is
20 associated with a faster rate of gain.
7. The method of claim 1 wherein the step of identifying the polymorphism is a method employing allele specific oligonucleotides.
- 25 8. The method of claim 1 wherein the step of identifying the polymorphism is selected from the group consisting of restriction fragment length polymorphism (RFLP) analysis, heteroduplex analysis, single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and use of linked genetic markers.

30

9. The method of claim 8 wherein the step of identifying the polymorphism comprises RFLP analysis.
10. The method of claim 1 further comprising the step of amplifying the MC4R gene
5 sequence.
11. The method of claim 10 further comprising the step of digesting the amplified region with the restriction endonuclease *Taq I*.
- 10 12. The amplified gene sequence of claim 10 wherein primers used in the amplification are selected from the group consisting of SEQ. ID NO:6, SEQ. ID NO:7, SEQ. ID NO:8, SEQ. ID NO:9, SEQ. ID NO:10, and SEQ. ID NO:11.
13. A single strand of an oligonucleotide primer useful for detecting nucleotide 678 of
15 the PCR product of a MC4R gene the primer consisting of a nucleotide sequence having about 4-30 contiguous bases from SEQ ID NO:1.
14. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:6.
- 20 15. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:7.
16. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide
25 sequence represented by SEQ ID NO:8.
17. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:9.
- 30 18. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:10.

19. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:11.

5 20. A method of identifying an animal which possess a desired genotype indicative of the metabolic traits of fat content, growth rate, and feed consumption, the method comprising

- a) obtaining a sample of genomic DNA,
- b) digesting the sample with *Taq I* to obtain fragments,
- 10 c) separating the fragments obtained from the digestion, and
- d) identifying the presence or absence of a *Taq I* site at base 678 of the PCR product of the MC4R gene.

21. The method of claim 20 further comprising the step of selecting animals with the
15 desired genotype for breeding.

22. The method of claim 20 wherein the site is identifiable by fragments of 466, 225, and 76 bp when a guanine is present at base 678 and fragments of 542 and 225 bp when an adenine is present when a restriction enzyme which cuts at the same recognition site as *Taq I* is used.
20

23. The method of claim 20 wherein the step of identifying comprises detecting the *Taq I* site by amplification.

25 24. A kit for evaluating a sample of animal DNA comprising a reagent in a container that identifies a polymorphism in a MC4R gene.

25. The kit of claim 24 wherein the reagent is a primer that amplifies the MC4R gene or a fragment thereof.

30

26. The kit of claim 24 further comprising a DNA polymerase which cleaves the MC4R gene, a forward primer, and a reverse primer, wherein the primers are capable of amplifying a region of the MC4R gene which contains a polymorphic site.
- 5 27. A primer for assaying the presence of a polymorphic *TaqI* site in the MC4R gene wherein the primer comprises a sequence selected from the group consisting of SEQ. ID NO:6, SEQ. ID NO:7, SEQ. ID NO:8, SEQ. ID NO:9, SEQ. ID NO:10, and SEQ. ID NO:11.
- 10 28. A method for selecting animals for the desired traits of lower fat content, faster growth rate, or lower feed consumption comprising the steps of
- a) obtaining a nucleic acid sample from an animal,
 - b) identifying a polymorphism characterized by a nucleotide position 678 of a PCR product of the MC4R gene, and
 - 15 c) selecting the animals which have the nucleotide associated with the desired traits in position 678.
29. A method for an indirect selection for a polymorphism in MC4R wherein specific alleles of an alternative DNA marker are used to make the indirect selection wherein the
- 20 alternative DNA marker is a linked marker near MC4R.
30. The method of claim 29 wherein the linked marker is selected from the group consisting of S0331, BHT0433, and S0313.
- 25 31. A method of identifying animals which possess a desired genotype indicative of the metabolic traits of fat content, growth rate, and feed consumption, the method comprising
- a) determining an association between a MC4R genotype and a trait of interest by obtaining a sample of animals from a line or breed of interest,
 - b) preparing genomic DNA from each animal in the sample,
 - 30 c) determining the genotype of the MC4R gene, and
 - d) calculating the association between the MC4R genotype and the trait.

32. A method of selecting animals which possess a desired MC4R genotype indicative of the metabolic traits of fat content, growth rate, and feed consumption, the method comprising

- 5 a) obtaining a nucleic acid sample from an animal,
- b) identifying the genotype of the MC4R gene of the animal, and
- c) selecting those animals which have the genotype associated with the desired traits.

FIGURE 1.

CONSENSUS SEQUENCE OF MC4R IN PIG

!!NA_SEQUENCE 1.0

con-mc4r.seq Length: 746 April 9, 1998 16:17 Type: N Check: 125

```
1  ACAAGAACTCT GCATTCACCC ATGTACTTTT TCATCTGTAG CCTGGCTGTG
51  GCTGATATGC TGGTGAGCGT TTCCAATGGG TCAGAAACCA TTGTCATCAC
101 CCTATTAAAC AGCACGGACA CGGACGCACA GAGTTTCACA GTGAATATTG
151 ATAATGTCAT TGA CTGAGTG ATCTGTAGCT CCTTACTCGC CTCAATTTGC
201 AGCCTGCTTT CGATTGCAGT GGACAGGTAT TTTACTATCT TTTATGCTCT
251 CCAGTACCAT AACATTATGA CAGTTAAGCG GGTGGAATC ATCATCAGTT
301 GTATCTGGGC AGTCTGCACG GTGTCGGGTG TTTGTTCAT CATTTACTCA
351 GATAGCAGTG CTGTTATTAT CTGCCTCATA ACCGTGTTCT TCACCATGCT
401 GGCTCTCATG GCTTCTCTCT ATGTCCACAT GTTCCTCATG GCCAGACTCC
451 ACATTAAGAG GATCGCCGTC CTCCCAGGCA CTGGCACCAT CCGCCAAGGT
501 GCCAACATGA AGGGGGCAAT TACCCTGACC ATCTTGATTG GGGTCTTTGT
551 GGTCTGCTGG GCCCCCTTCT TCCTCCACTT AATATTCTAT ATCTCCTGCC
601 CCCAGAATCC ATACTGTGTG TGCTTCATGT CTCACTTTAA TTTGTATCTC
651 ATCCTGATCA TGTGTAATTC CATCATCAT CCCCTGATT ATGCACTCCG
701 GAGCCAAGAA CTGAGGAAAA CCTTCAAAGA GATCATCTGT TGCTAT
```

X = G or A, the site of polymorphism.

FIGURE 2.

COMPARISON OF DNA SEQUENCE BETWEEN HUMAN AND PORCINE MC4R GENE

(Nucleotide) FASTA of: con-mc4r.seq from: 1 to: 746 April 10, 1998 19:52

TO: s77415.gb_pr Sequences: 1 Symbols: 1,671 Word Size: 6

Searching with both strands of the query.

Scoring matrix: GenRunData:fastadna.cmp

Constant pamfactor used

Gap creation penalty: 16 Gap extension penalty: 4

The best scores are:

initl initn opt..

/usr2/users/rothschi/kwan/mc4r/seq/s77415.gb_pr Begin: 608 End: 1353

! LOCUS S77415 1671 bp ... 3208 3208 3208

/usr2/users/rothschi/kwan/mc4r/seq/s77415.gb_pr Begin: 388 End: 407

Strand: -

! LOCUS S77415 1671 bp ... 70 70 73

\\End of List

con-mc4r.seq

/usr2/users/rothschi/kwan/mc4r/seq/s77415.gb_pr

LOCUS S77415 1671 bp DNA PRI 26-SEP-1995

DEFINITION melanocortin-4 receptor [human, Genomic, 1671 nt].

ACCESSION S77415

NID g998456

KEYWORDS

SOURCE human. . . .

SCORES Initl: 3208 Initn: 3208 Opt: 3208

92.2% identity in 746 bp overlap

(SEQ ID NO:3)

con-mc4r.seq

10 20 30
ACAAGAATCTGCATTACCCATGTACTTTT
|||||

s77415

ATATCTTAGTGATTGTGGCAATAGCCAAGACCAAGAATCTGCATTACCCATGTACTTTT
580 590 600 610 620 630

(SEQ ID NO:2)

40 50 60 70 80 90
con-mc4r.seq TCATCTGTAGCCTCCTGTGGCTGATATGCTGGTGAGCGTTTCCAATGGGTGAGAAACCA
||||| ||| ||||| ||||| ||||| ||||| |||||

s77415

TCATCTGCAGCTTGGCTGTGGCTGATATGCTGGTGAGCGTTTCCAATGGGTGAGAAACCA
640 650 660 670 680 690

100 110 120 130 140 150
con-mc4r.seq TTGTATCACCCTATTAAACAGCAGCGGACGCGGACGACAGAGTTTCACAGTGAATATTG
|| ||||| ||||| || ||||| ||||| ||||| |||||

s77415

TTATCATCACCCTATTAAACAGTACAGATACGGATGCACAGAGTTTCACAGTGAATATTG
700 710 720 730 740 750

FIGURE 2. (cont.)

```

      160      170      180      190      200      210
con-mc4r.seq ATAATGTCATTGACTCAGTGATCTGTAGCTCCTTACTCGCCTCAATTTCAGCCTGCTTT
s77415      ATAATGTCATTGACTCGGTGATCTGTAGCTCCTTGCCTTCATCCATTTCAGCCTGCTTT
      760      770      780      790      800      810

      220      230      240      250      260      270
con-mc4r.seq CGATTGCAGTGGACAGGTATTTTACTATCTTTTATGCTCTCCAGTACCATAACATATGA
s77415      CAATTGCAGTGGACAGGTACTTTTACTATCTTCTATGCTCTCCAGTACCATAACATTATGA
      820      830      840      850      860      870

      280      290      300      310      320      330
con-mc4r.seq CAGTTAAGCGGGTTGGAATCATCATCAGTTGTATCTGGGCAGTCTGCACGGTGTGGGGTG
s77415      CAGTTAAGCGGGTTGGGATCAGCATAAGTTGTATCTGGGCAGCTTGCACGGTTTCAGGCA
      880      890      900      910      920      930

      340      350      360      370      380      390
con-mc4r.seq TTTTGTTCATCATTTACTCAGATAGCAGTGCTGTTATTATCTGCCTCATACCGTGTCT
s77415      TTTTGTTCATCATTTACTCAGATAGTAGTGCTGTCATCATCTGCCTCATCACCATGTCT
      940      950      960      970      980      990

      400      410      420      430      440      450
con-mc4r.seq TCACCATGCTGGCTCTCATGGCTTCTCTATGTCCACATGTTCCATGGCCAGACTCC
s77415      TCACCATGCTGGCTCTCATGGCTTCTCTATGTCCACATGTTCCATGGCCAGGCTTC
      1000     1010     1020     1030     1040     1050

      460      470      480      490      500      510
con-mc4r.seq ACATTAAGAGGATCGCCGCTCTCCAGGCACTGGCACCATCCGCCAAGGTGCCAATATGA
s77415      ACATTAAGAGGATTCCTGTCTCTCCCGGCACTGGTGCCATCCGCCAAGGTGCCAATATGA
      1060     1070     1080     1090     1100     1110

      520      530      540      550      560      570
con-mc4r.seq AGGGGGCAATTACCTGACCATCTTGATTGGGGTCTTTGTGGTCTGCTGGGCCCCCTTCT
s77415      AGGGAGCGATTACCTTGACCATCTTGATTGGGCTCTTTGTGTCTGCTGGGCCCCATCTT
      1120     1130     1140     1150     1160     1170

      580      590      600      610      620      630
con-mc4r.seq TCCTCCACTTAATATTCTATATCTCTGCCCCAGAAATCCATCTGTGTGCTTCATGT
s77415      TCCTCCACTTAATATTCTATATCTCTGCCCCAGAAATCCATATTGTGTGCTTCATGT
      1180     1190     1200     1210     1220     1230

      640      650      660      670      680      690
con-mc4r.seq CTCACTTTAATTGTATCTCATCTGATCATGTGTAATCCATCATCAATCCCGTGATT
s77415      CTCACTTTAATTGTATCTCATCTGATCATGTGTAATCAATCATCATCTCTGATT
      1240     1250     1260     1270     1280     1290

      700      710      720      730      740
con-mc4r.seq ATGCACTCCGGAGCCAAGAACTGAGGAAAACCTTCAAAGAGATCATCTGTTGCTAT
s77415      ATGCACTCCGGAGTCAAGAACTGAGGAAAACCTTCAAAGAGATCATCTGTTGCTATCCCC
      1300     1310     1320     1330     1340     1350

s77415      TGGGAGGCCCTTTGTGACTTGTCTAGCAGATATTAAATGGGGACAGAGCACGCAATATAGG
      1360     1370     1380     1390     1400     1410

```

FIGURE 3.

COMPARISON OF AMINO ACID SEQUENCE BETWEEN HUMAN AND
PORCINE MC4R GENE

(Peptide) TFASTA of: human.pep from: 1 to: 332 April 9, 1998 16:18

REFORMAT of: human check: 9754 from: 1 to: 332 March 7, 1998 18:43
(No documentation)

TO: mc4r-allele*.seq Sequences: 2 Symbols: 1,492 Word Size: 2

Searching all six frames.

Scoring matrix: GenRunData:blosun50.cmp

Variable pamfactor used

Gap creation penalty: 16 Gap extension penalty: 4

The best scores are: frame init1 initn opt..

```

/usr2/users/rothschi/kwan/mc4r/seq/mc4r-allele1.seq Begin: 3 End: 746
! mc4r-allele1.seq Length: 746 April ... (3) 1602 1602 1602
/usr2/users/rothschi/kwan/mc4r/seq/mc4r-allele2.seq Begin: 3 End: 746
! mc4r-allele2.seq Length: 746 April ... (3) 1596 1596 1596

```

SCORES Frame: (3) Init1: 1602 Initn: 1602 Opt: 1602
97.6% identity in 248 aa overlap

```

(SEQ ID NO:4)      50      60      70      80      90     100
human.pep  QLFVSPEVFTLGVISLLENILVIVAIKKNLHSPMYFFICSIAVADMLVSVSNGSETI
              |||||
mc4r-allele1      KNLHSPMYFFICSIAVADMLVSVSNGSETI
(SEQ ID NO:5)      10      20      30

              110      120      130      140      150      160
human.pep  IITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMT
              :|||
mc4r-allele1 VITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMT
              40      50      60      70      80      90

              170      180      190      200      210      220
human.pep  VKRVGISISCIWAACVSGILFIIYSDSSAVIICLITMFFTMLALMASLYVHMFIMARLH
              |||||
mc4r-allele1 VKRVGIIISCIWAVCTVSGVLFIIYSDSSAVIICLITVFFTMLALMASLYVHMFIMARLH
              100      110      120      130      140      150

              230      240      250      260      270      280
human.pep  IKRIAVLPGTGAIROGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMS
              |||||
mc4r-allele1 IKRIAVLPGTGTIROGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMS
              160      170      180      190      200      210

              290      300      310      320      330
human.pep  HFNLYLILIMCNSIIDPLIYALRSQELRKTFKEIICCYPLGGLCDLSSRY
              |||||
mc4r-allele1 HFNLYLILIMCNSIIDPLIYALRSQELRKTFKEIICCY
              220      230      240

```

FIGURE 3. (cont.)

human.pep
 /usr2/users/rothschi/kwan/mc4r/seq/mc4r-allele2.seq

SCORES Frame: (3) Initl: 1596 Inith: 1596 Opt: 1596
 97.28 identity in 248 aa overlap

	50	60	70	80	90	100
human.pep	QLFVSPEVFTLGVISILENILVIVAIAKKNLHSPMYFFICSLAVADMLVSVSNGSETI					
mc4r-allele2	KNLHSPMYFFICSLAVADMLVSVSNGSETI					
		10	20	30		
	110	120	130	140	150	160
human.pep	IITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMT					
	:					
mc4r-allele2	VITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMT					
	40	50	60	70	80	90
	170	180	190	200	210	220
human.pep	VKRVGISISCIWAAC TVSGILFIIYSDSSAVIICLITMFFTMLALMASLYVHMFIMARLH					
	: : : :					
mc4r-allele2	VKRVGIIISCIWAVCTVSGVLFIIYSDSSAVIICLITVEFTMLALMASLYVHMFIMARLH					
	100	110	120	130	140	150
	230	240	250	260	270	280
human.pep	IKRIAVLPGTGAIRQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMS					
	: : : :					
mc4r-allele2	IKRIAVLPGTGTIRQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMS					
	160	170	180	190	200	210
	290	300	310	320	330	
human.pep	HFNLYLILIMCNSIIDPLIYALRSQELRKTTFKEIICCYPLGGICDLSSRY					
mc4r-allele2	HFNLYLILIMCNSIINPLIYALRSQELRKTTFKEIICCY					
	220	230	240			

! CPU time used:
 ! Database scan: 0:00:00.2
 ! Post-scan processing: 0:00:00.1
 ! Total CPU time: 0:00:00.4
 ! Output File: human.tfasta

FIGURE 4a.

LINKAGE REPORT OF TWO-POINT ANALYSIS

New locus MC4R
in sex-equal linkage analyses with existing loci

S0082	MC4R	rec. fracs.=	0.05,	lods =	14.74
CGA	MC4R	rec. fracs.=	0.14,	lods =	6.88
S0020	MC4R	rec. fracs.=	0.18,	lods =	5.32
S0079	MC4R	rec. fracs.=	0.12,	lods =	10.35
S0155	MC4R	rec. fracs.=	0.14,	lods =	7.68
S0122	MC4R	rec. fracs.=	0.18,	lods =	5.17
S0313	MC4R	rec. fracs.=	0.00,	lods =	17.76
S0312	MC4R	rec. fracs.=	0.20,	lods =	5.60
S0311	MC4R	rec. fracs.=	0.17,	lods =	7.18
S0416	MC4R	rec. fracs.=	0.20,	lods =	3.21
S0331	MC4R	rec. fracs.=	0.02,	lods =	21.91
S0396	MC4R	rec. fracs.=	0.16,	lods =	7.85
BHT0433	MC4R	rec. fracs.=	0.02,	lods =	21.32
S0536	MC4R	rec. fracs.=	0.03,	lods =	15.61
CAPN3	MC4R	rec. fracs.=	0.12,	lods =	6.65
KGF	MC4R	rec. fracs.=	0.09,	lods =	6.46
MEF2A	MC4R	rec. fracs.=	0.05,	lods =	14.36
MC4R	MC4R	rec. fracs.=	0.00,	lods =	26.19

FIGURE 4b.

new locus MC4R

in sex-specific (female/male) linkage analyses with existing loci

S0082	MC4R	rec. fracs.=	0.00	0.09,	lods =	15.86
CGA	MC4R	rec. fracs.=	0.07	0.22,	lods =	7.46
S0020	MC4R	rec. fracs.=	0.00	0.25,	lods =	6.33
S0079	MC4R	rec. fracs.=	0.00	0.19,	lods =	11.48
S0155	MC4R	rec. fracs.=	0.00	0.24,	lods =	9.98
S0122	MC4R	rec. fracs.=	0.00	0.27,	lods =	7.10
S0313	MC4R	rec. fracs.=	0.00	0.00,	lods =	17.76
S0312	MC4R	rec. fracs.=	0.04	0.29,	lods =	7.45
S0311	MC4R	rec. fracs.=	0.00	0.28,	lods =	9.02
S0416	MC4R	rec. fracs.=	0.00	0.31,	lods =	4.17
S0331	MC4R	rec. fracs.=	0.05	0.00,	lods =	22.14
S0396	MC4R	rec. fracs.=	0.03	0.24,	lods =	9.33
BHT0385	MC4R	rec. fracs.=	0.14	0.36,	lods =	3.46
BHT0433	MC4R	rec. fracs.=	0.05	0.00,	lods =	21.82
S0536	MC4R	rec. fracs.=	0.00	0.05,	lods =	15.77
CAPN3	MC4R	rec. fracs.=	0.00	0.18,	lods =	7.35
KGF	MC4R	rec. fracs.=	0.00	0.17,	lods =	6.74
MEF2A	MC4R	rec. fracs.=	0.10	0.00,	lods =	14.52
MC4R	MC4R	rec. fracs.=	0.00	0.00,	lods =	26.19

FIGURE 4c.

LINKAGE REPORT OF MULTIPOINT ANALYSIS

Sex_averaged map (recomb. frac., Kosambi cM):

0	ESR			0.0
		0.18	18.4	
1	S0008			18.4
		0.12	11.9	
7	CGA			30.3
		0.03	2.8	
3	S0312			33.1
		0.05	4.9	
4	S0122			38.1
		0.09	9.4	
8	KGF			47.4
		0.06	5.8	
6	CAPN3			53.2
		0.02	2.5	
9	MEF2A			55.7
		0.06	6.1	
5	MC4R			61.8
		0.06	5.6	
10	S0313			67.4
		0.00	0.0	
11	S0082			67.4
		0.03	3.4	
12	S0079			70.8
		0.03	2.5	
14	S0142			73.3
		0.01	1.0	
13	S0020			74.4
		0.04	4.3	
15	S0311			78.7
		0.00	0.0	
16	S0155			78.7
		0.12	12.2	
17	S0113			90.9
		0.20	21.0	
18	S0302			111.9
		0.22	23.4	
19	S0112			135.3

* denotes recomb. frac. held fixed in this analysis. log10_like = -305.098

FIGURE 6.

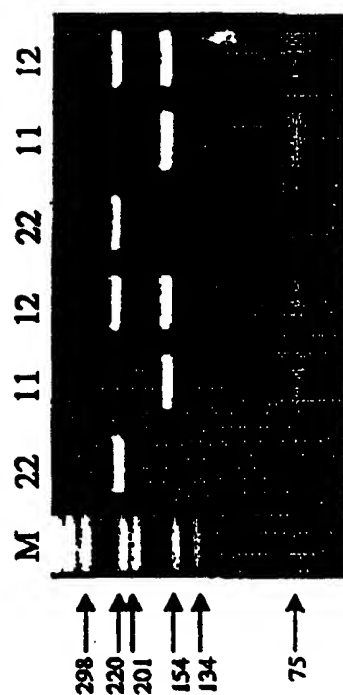
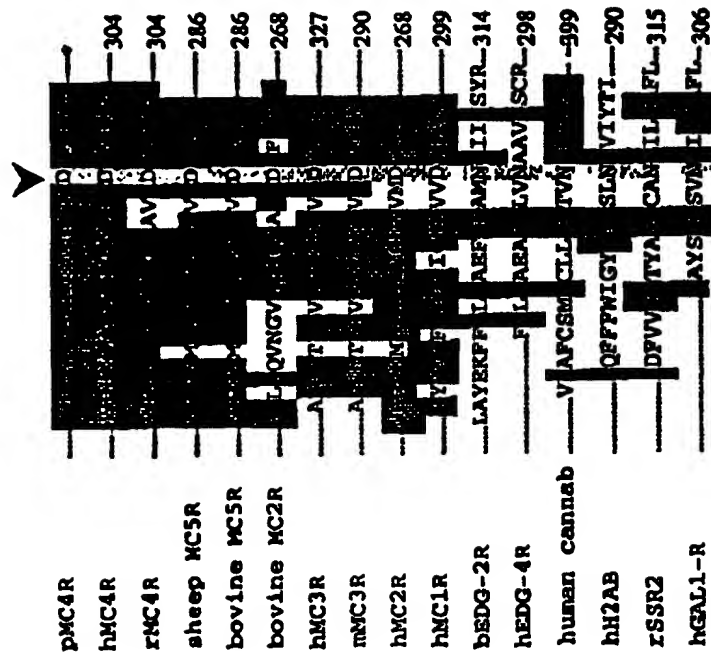


FIGURE 7.



PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(72) Inventors; and (75) Inventors/Applicants (for US only): ROTHSCHILD, Max, F. [US/US]; Office of Intellectual Property & Technology Transfer, 310 Lab of Mechanics, Ames, IA 50011-2131 (US). LARSON, Niels, J. [DK/DK]; Office of Intellectual Property & Technology Transfer, 310 Lab of Mechanics, Ames, IA 50011-2131 (US). KIM, Kwan, Suk [US/US]; Office of Intellectual Property & Technology Transfer, 310 Lab of Mechanics, Ames, IA 50011-2131 (US).		(88) Date of publication of the international search report: 11 May 2000 (11.05.00)	
(54) Title: MELANOCORTIN-4 RECEPTOR GENE AND USE AS A GENETIC MARKER FOR FAT CONTENT, WEIGHT GAIN, AND/OR FEED CONSUMPTION OF ANIMALS			
(57) Abstract <p>Genetic markers in the porcine melanocortin-4 receptor (MC4R) gene are disclosed which are associated with fat content, growth rate, and feed consumption. Further, novel sequence data from regions of the gene are disclosed which may be used in a PCR test to screen for the presence of the marker. The genetic marker may be used to screen animals for breeding purposes which have the desired traits regarding fat content, growth rate, and feed consumption. Kits which take advantage of the PCR test are also disclosed.</p>			

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/16862

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12Q1/68 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GOTODA T ET AL: "Molecular screening of the human melanocortin-4 receptor gene: identification of a missense variant showing no association with obesity, plasma glucose, or insulin" DIABETOLOGIA, vol. 40, no. 8, August 1997 (1997-08), pages 976-79, XP000866534 ---	
A	ANDERSSON L ET AL: "GENETIC MAPPING OF QUANTITATIVE TRAIT LOCI FOR GROWTH AND FATNESS IN PIGS" SCIENCE, US, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 263, page 1771-1774 XP002018359 ISSN: 0036-8075 --- -/--	

☒ Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 23644 A (COFOK B V ;BEHEERMAATSCHAPPIJ VARKENSVERB (NL); INSTITUUT VOOR DIE) 3 July 1997 (1997-07-03) ---	
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information on patent family members

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